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Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats

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Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. The 5/6 nephrectomy model is used to study pathogenetic mechanisms underlying chronic renal failure. We previously demonstrated that increased mesangial cell proliferation and glomerular PDGF B-chain expression precede glomerulosclerosis in this model. In the present study we have assessed the concomitant changes in the cortical tubulointerstitium. A wave of tubular and interstitial cell proliferation (as determined by immunostaining for PCNA) occurred at week 1 after 5/6 nephrectomy. This wave preceded the peak glomerular cell proliferation by one week. Tubulointerstitial cell proliferation decreased thereafter and reached control values by week 10. *In situ* hybridization and immunostaining for PDGF B-chain and β-receptor in sham-operated controls showed labeling of distal tubules and collecting ducts, while no signal was present in the interstitium. PDGF B-chain mRNA and protein expression was markedly increased in tubules at weeks 2 and 4 after 5/6 nephrectomy and in the interstitium (particularly in areas of inflammatory infiltrates) at weeks 2 to 10. Similar changes occurred with PDGF receptor β-subunit immunostaining. Interstitial expression of desmin and α-smooth muscle actin (markers of myofibroblasts) progressively increased after week 1. Interstitial influx of monocytes/macrophages with focal accentuation started at week 2. Counts of lymphocytes, neutrophils and platelets showed only minor changes. In parallel to the monocyte/macrophage influx, progressive interstitial accumulation of collagens I and IV, laminin, and fibronectin occurred. All of these changes were correlated with the increase in serum creatinine, proteinuria and an index of tubulointerstitial damage. We conclude that tubulointerstitial changes after 5/6 nephrectomy show similarities with those observed in the glomeruli. Tubular and interstitial overexpression of PDGF B-chain and its receptor may play a role in mediating fibroblast migration and/or proliferation in areas of tubulointerstitial injury.

The development of progressive renal dysfunction even after the apparent resolution of an injurious condition is observed in a large number of renal diseases [1]. The most frequently employed model to study the events that follow extensive loss of functioning renal tissue, is the 5/6 nephrectomy model [2]. In this model, adaptive renal hypertrophy and hyperplasia quickly follow the infarction or ligation of 5/6 of the kidneys [3–6]. Progressive glomerulosclerosis, tubulointerstitial injury, renal dysfunction, and, ultimately, uremia develop thereafter [2–6]. Earlier studies on the mechanisms underlying this progressive renal damage have focused on the pathogenesis of glomerulosclerosis [3–6]. In an extension of these studies, we have demonstrated recently that 5/6 nephrectomy in the rat is followed by an early wave of glomerular mesangial cell proliferation which may have been induced and/or maintained by the concomitant glomerular overexpression of platelet-derived growth factor (PDGF) B-chain and up-regulation of PDGF receptor β-subunit [7]. Also, among the very early events we noted a phenotypic switch of mesangial cells, in which these cells acquired characteristics of a cell type involved in scar formation, namely the myofibroblast [7]. All of these changes preceded expansion of the glomerular extracellular matrix, glomerular influx of monocytes/macrophages, and glomerulosclerosis [7, 8].

It has repeatedly been suggested that primary or secondary tubulointerstitial injury may be of equal or greater importance than glomerular changes in determining whether progressive renal dysfunction will ensue [9, 10]. Subsequently, a strong correlation of tubulointerstitial damage with reduced renal function has been demonstrated to occur in various immunological glomerular diseases, including membranous nephropathy [11], mesangioproliferative glomerulonephritis [12], focal segmental glomerulosclerosis [13], type I mesangiocapillary glomerulonephritis [14], and lupus nephritis [15] as well as in non-immune glomerular diseases, such as diabetic nephropathy [16]. To better understand the pathogenic mechanisms leading to renal interstitial injury and in particular the relationship between glomerular and tubulointerstitial injury, we have assessed in the same renal biopsies that had been evaluated previously for glomerular changes (see above) [7, 8] the sequence of tubulointerstitial changes after 5/6 nephrectomy. Specifically, we have addressed the questions of whether or not: (1) tubulointerstitial cell proliferation and influx of inflammatory cells parallel the changes observed in the glomeruli; (2) a phenotypic change from interstitial fibroblasts to myofibroblasts similar to the phenotypic change observed in mesangial cells occurs; (3) there is evidence to suggest an involvement of PDGF in any of the aforementioned changes; and (4) how the aforementioned changes relate to the development of interstitial matrix accumulation, proteinuria, and tubulointerstitial damage.

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Methods

Disease model and experimental design

As described previously in more detail [7], 70 male Sprague-Dawley rats (Simonson, Gilroy, CA, USA) weighing 140 to 160 g at the start of the experiment were studied. In 35 rats (OP group) a right subcapsular nephrectomy and infarction of approximately two-thirds of the left kidney was accomplished by ligation of the posterior and one or two anterior extrarenal branches of the main renal artery. In the control group (SHAM) of 35 rats, a sham operation consisting of laparotomy and manipulation of the renal pedicles but without destruction of renal tissue was performed. Postoperative 24-hour urinary protein excretion and serum creatinine values were determined at days 3 and 5 as well as at weeks 1, 2, 3, 4, 7.5 and 10 in eight to ten randomly selected animals of both groups. At each time point five rats from each group underwent renal biopsy (no rat underwent more than one biopsy). At 10 weeks the surviving rats (31 of the OP group and 35 of the SHAM group) were sacrificed and further renal tissue specimens were obtained. In all cases cortical biopsies were taken from the center of the non-infarcted area only. Biopsies were studied to assess cortical tubulointerstitial injury, proliferating tubulointerstitial cells (as defined by staining for the proliferating cell nuclear antigen, PCNA), monocytes/macrophages, neutrophils, lymphocytes, platelets, cells expressing α-smooth muscle actin and/or desmin, PDGF B-chain (protein and mRNA), PDGF receptor β-subunit, and extracellular matrix proteins including type I and IV collagen, laminin, and fibronectin.

Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [17] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Tubulointerstitial injury was defined as inflammatory cell infiltrates, tubular dilatation and/or atrophy, or interstitial fibrosis. Injury was graded by an observer, who was unaware of the origin of the slides, according to Shih, Hines and Neilson [18] on a scale of 0 to 4 (0 = normal; 0.5 = small focal areas of damage; 1 = involvement of less than 10% of the cortex; 2 = involvement of 10 to 25% of the cortex; 3 = involvement of 25 to 75% of the cortex; 4 = extensive damage involving more than 75% of the cortex).

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by a direct or indirect immunoperoxidase technique as previously described [17]. Primary antibodies included:

- 19A2 (American Biotech Inc., Plantation, FL, USA), a murine IgM monoclonal antibody against human PCNA, which is expressed by actively proliferating cells. Nuclear expression of PCNA is absent in the G_0 and early G_1 phase of the cell cycle and increases in the late G_1 and S phase, followed by a decrease in G_2/M [19]. We have previously shown in angiotensin-II infused rats, that cell proliferation as determined by this anti-PCNA antibody correlates with the cell proliferation as assessed by the conventional method of ^{3}H-thymidine incorporation [20].
- ED1 (Bioproducts for Science, Indianapolis, IN, USA), a murine monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells [21].
- RP-3 (gift of F. Sendo, Yamagata, Japan), a murine monoclonal IgM antibody to rat neutrophils [22].
- OX-22 (Accurate Chemical Corporation, Westbury, NY, USA), a murine monoclonal IgG antibody to the high molecular weight form of the rat common leukocyte antigen expressed on B-lymphocytes and some T-lymphocytes.
- PL-1, a murine monoclonal antibody against rat platelets (gift of W.W. Bakker, Groningen, The Netherlands) [23].
- PGF-007 (Mochida Pharmaceutical, Tokyo, Japan), a murine monoclonal antibody to a 25 amino acid peptide located near the COOH-terminus of the human PDGF B-chain (gift of Mochida Pharmaceutical, Tokyo, Japan) [24].
- A rabbit polyclonal antibody to the β-subunit of the PDGF-receptor as described elsewhere [25] (gift of R. Seifert and D. Bowen-Pope, Seattle, WA, USA).
- α-SM1, a murine monoclonal antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin (gift of G. Gabbiani, Geneva, Switzerland) [26].
- D33, a murine monoclonal IgG_1 antibody against human muscle desmin (Dako, Glostrup, Denmark).
- An IgG fraction of polyclonal guinea pig anti-rat type I collagen [27] (provided by L. Iruela-Arispe, Seattle WA, USA).
- Affinity purified polyclonal goat anti-human/bovine type IV collagen (Southern Biotechnology, Birmingham, AL, USA).
- An IgG fraction of polyclonal rabbit anti-rat laminin (Chemicon, Temecula, CA, USA).
- An affinity purified IgG fraction of polyclonal rabbit anti-rat fibronectin (Chemicon).

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit or goat IgG. Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

To obtain mean numbers of proliferating cells or infiltrating leukocytes in the cortical tubulointerstitium over 20 grid fields (range 20 to 60), measuring 0.1 mm^2 each, were analyzed (grid parts containing glomerular cross sections were ignored in this evaluation) and mean counts per biopsy were obtained. Proliferating cells in the tubulointerstitium were further separated into those, which localized to a tubular cross section with a lumen ("tubular cells") and those, which localized to any other area ("other cells"). This latter group represents a mixture of interstitial cells and vascular cells. Furthermore, since it was not possible in any case to clearly identify a tubular basement membrane in the immunostained sections, the "other cells" group is likely to contain an unknown percentage of cells belonging to tubules, which had been sectioned tangentially.

To analyze whether the biased method [28] of counting cell numbers in a single section yields comparable results as an unbiased method, we evaluated PCNA positive nucleus counts in the tubulointerstitium of some additional 5/6 nephrectomized rats ($N = 5$) using either the method described above or in two serial sections using the disector principle [29]. The data showed that the counts of PCNA positive nuclei per mm^2 in a single section were highly correlated with those of PCNA positive nuclei per mm^3 as determined by the disector ($r = 0.93$, $P < 0.01$).

For the evaluation of the immunoperoxidase stains for α-smooth muscle actin and desmin, each tubulointerstitial grid field was graded semiquantitatively, and the mean score per biopsy was calculated. Each score mainly reflected changes in the

extent rather than intensity of tubulointerstitial staining and depended on the percentage of the grid field showing positive staining: 0 = absent staining, I = 1 to 5%, II = 5 to 25%, III = 25 to 50%, IV = 50 to 75%, V = >75%. To test the validity of this scoring system, all α-smooth muscle actin stains were scored independently by two different observers, both of whom were unaware of the origin of the sections. Furthermore, all sections were evaluated by morphometry using the IMAGEC software (Imtronic GmbH, Berlin, Germany), and the relative tubulointerstitial area exhibiting positive staining per mm^2 was determined in each specimen. The data (not shown) demonstrated that the semiquantitative scores obtained by the two reviewers were highly correlated ($r = 0.97$; $y = 0.99x + 0.03$; $P < 0.001$), as were the mean semiquantitative scores and the morphometric data ($r = 0.95$; $y = 1.05x + 2.1$; $P < 0.001$).

In the case of immunostaining for α-smooth muscle actin and desmin, the periglomerular areas were assessed separately. Semiquantitative staining scores in these cases depended on the percentage of the interstitium immediately contiguous to Bowman's capsule showing positive staining: 0 = No part stained, I = 1 to 5%, II = 5 to 25%, III = 25 to 50%, IV = 50 to 75%, V = > 75%.

Immunohistochemical double-staining

Double immunostaining for the identification of the type of proliferating cells was performed as reported previously [25] by first staining the sections for proliferating cells with 19A2, an IgM monoclonal antibody to PCNA, using an indirect immunogold procedure. Sections were then incubated with the IgG_1 monoclonal antibodies ED1, a monocyte/macrophage marker, or α-SM1, which is directed against α-smooth muscle actin (expressed by vascular smooth muscle cells and myofibroblasts [30]). Cells were identified as proliferating monocytes/macrophages or vascular smooth muscle cells/myofibroblasts if they showed positive nuclear staining for PCNA and if the nucleus was completely surrounded by cytoplasm positive for either ED1 (monocytes/macrophages) or α-smooth muscle actin (vascular smooth muscle cells and myofibroblasts). Proliferating cells in which the PCNA positive nucleus did not border on a cytoplasm positive for ED1 or α-smooth muscle actin were classified as non-monocyte/macrophage or non-vascular smooth muscle cell/myofibroblast. Proliferating (PCNA+) cells which could not be clearly identified as ED1 or α-smooth muscle actin positive or negative were considered non-classifiable. Negative controls included omission of either of the primary antibodies in which case no double-staining was noted.

In situ hybridization for PDGF B-chain

In situ hybridization was performed on 4 μm sections of biopsy tissue fixed in buffered 10% formalin utilizing a digoxigenin-labeled anti-sense cRNA probe for the murine PDGF B-chain (derived from a cDNA provided by Charles Stiles, Boston, MA, USA) as described [31]. Detection of the cRNA probe was performed with an alkaline phosphatase coupled anti-digoxigenin antibody (Genius Nonradioactive Nucleic Acid Detection Kit, Boehringer-Mannheim, Mannheim, Germany) with subsequent color development. Controls consisted of hybridization with a sense probe to matched serial sections, by hybridization of the anti-sense probe to tissue sections which had been incubated with RNAse A before hybridization, or by deletion of the probe, antibody or color solution [31].

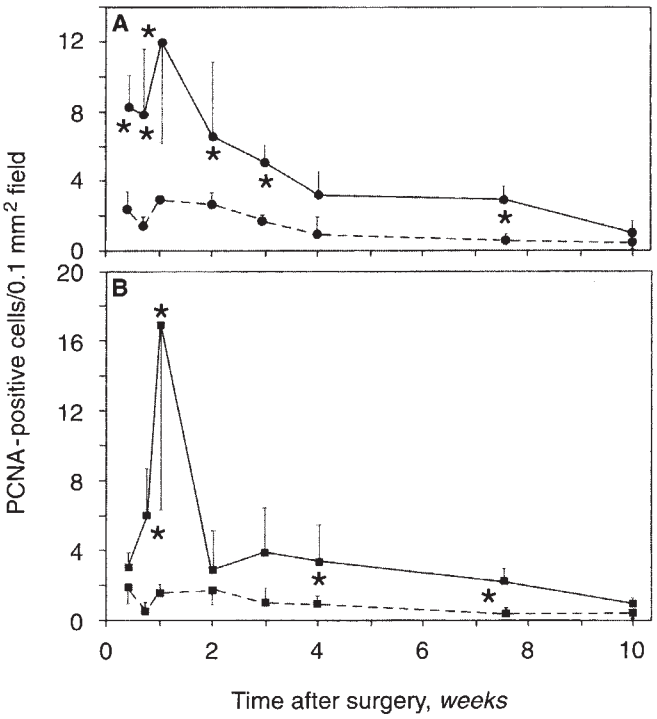


Fig. 1. *Cell proliferation (as defined by PCNA positive nuclear staining) in the tubulointerstitium after 5/6 nephrectomy or sham operation.* PCNA positive cells were subdivided according to their location as either localizing to a tubular cross section with a lumen (**A**) or to any other area (**B**) (see **Methods**). *$P < 0.05$ versus sham operated controls at the same time point. Symbols are: (solid line) 5/6 nephrectomy; (dashed line) sham operated.

Identification of tubular segments

We have characterized the distribution of immunohistochemical localizations of PDGF B-chain (protein and mRNA) and PDGF receptor β-subunit by careful delineation of morphologic features of the tubule segments exhibiting immunoreactivity. Distal tubules and collecting ducts were identified as tubular segments lined by cuboidal cells with large nuclear/cytoplasmic volume ratios which lacked brush borders. Proximal tubules were identified as segments lined by columnar cells with smaller nuclear/cytoplasmic volume ratios and which had swollen but identifiable brush boders.

Miscellaneous measurements

Serum creatinine was measured using picric acid (Worthington Diagnostics, Freehold, NJ, USA) and urine protein excretion was measured by a sulfosalicylic acid method [32] using a whole serum standard (Lab Trol, Dade Diagnostics, Aquado, Puerto Rico).

Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's *t*-test or one way analysis of variance with modified *t*-tests performed using the Bonferroni correction [33].

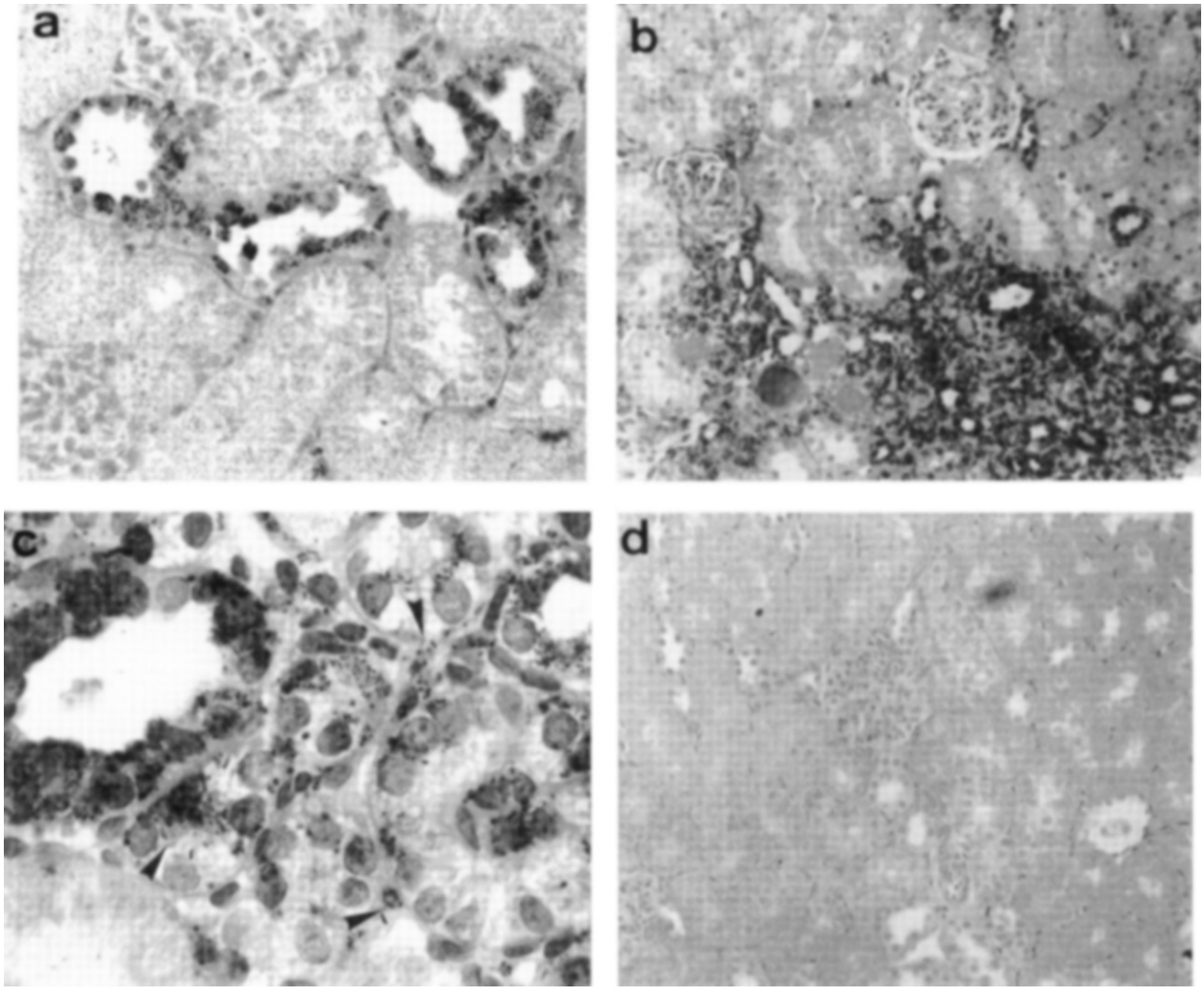


Fig. 2. *In situ hybridization for PDGF B-chain mRNA.* (**a**) Sham operated controls. Positive signal is present in distal tubules and collecting ducts (magnification ×400). (**b**) Week 2 after 5/6 nephrectomy. Up-regulated signal is present in a focal area (× 200). (**c**) Week 4 after 5/6 nephrectomy. Expression of PDGF B-chain mRNA is up-regulated in both tubular and interstitial cells (arrows; × 1000). (**d**) Tissue hybridized with a sense probe for PDGF B-chain mRNA does not exhibit signal (× 200).

Results

Proliferation: An early, transient wave of cortical tubulointerstitial cell proliferation follows 5/6 nephrectomy

In sham operated rats, low grade proliferative activity was noted in the cortical tubulointerstitium, which did not change during the time period of the study (Fig. 1). In contrast, in 5/6 nephrectomized rats a dramatic, diffuse increase in the number of both proliferating tubular cells and other cells occurred within three days after the operation (Fig. 1). Proliferative activity peaked at day 7 after the operation, then abruptly decreased and reached control values at week 10 (Fig. 1). By morphological criteria (such as height and density of the cells and the presence of a brush border) tubular proliferating cells appeared to localize to mainly to proximal tubules, but could also be detected in distal tubules and collecting ducts.

Double immunostaining was performed to assess the contribution of proliferating monocytes/macrophages and vascular smooth muscle cells or myofibroblasts to the proliferative activity in the cortical tubulointerstitium. In sham operated rats, virtually no proliferating monocytes/macrophages or vascular smooth muscle cells were detected at any time point. Following 5/6 nephrectomy, proliferating monocytes/macrophages were very rare and accounted for $< 3\%$ of the PCNA-positive cells at any time point. Proliferating vascular smooth muscle cells were also rare after 5/6 nephrectomy, except at the peak of the proliferative activity at day 7, when $3.8 \pm 1.0\%$ of the proliferating cells localized to smooth muscle cells in vessel walls. Proliferating myofibroblasts (that is, PCNA+/α-smooth muscle actin+ cells outside of vessels) could not be demonstrated in sham operated rats or in 5/6 nephrectomized rats prior to week 3 after 5/6 nephrectomy. In contrast, rare PCNA+/α-smooth muscle actin+ cells (accounting for $< 2\%$ of the total PCNA-positive cells) were noted in the interstitium, particularly the periglomerular interstitium, at week 3 and later time points after 5/6 nephrectomy.

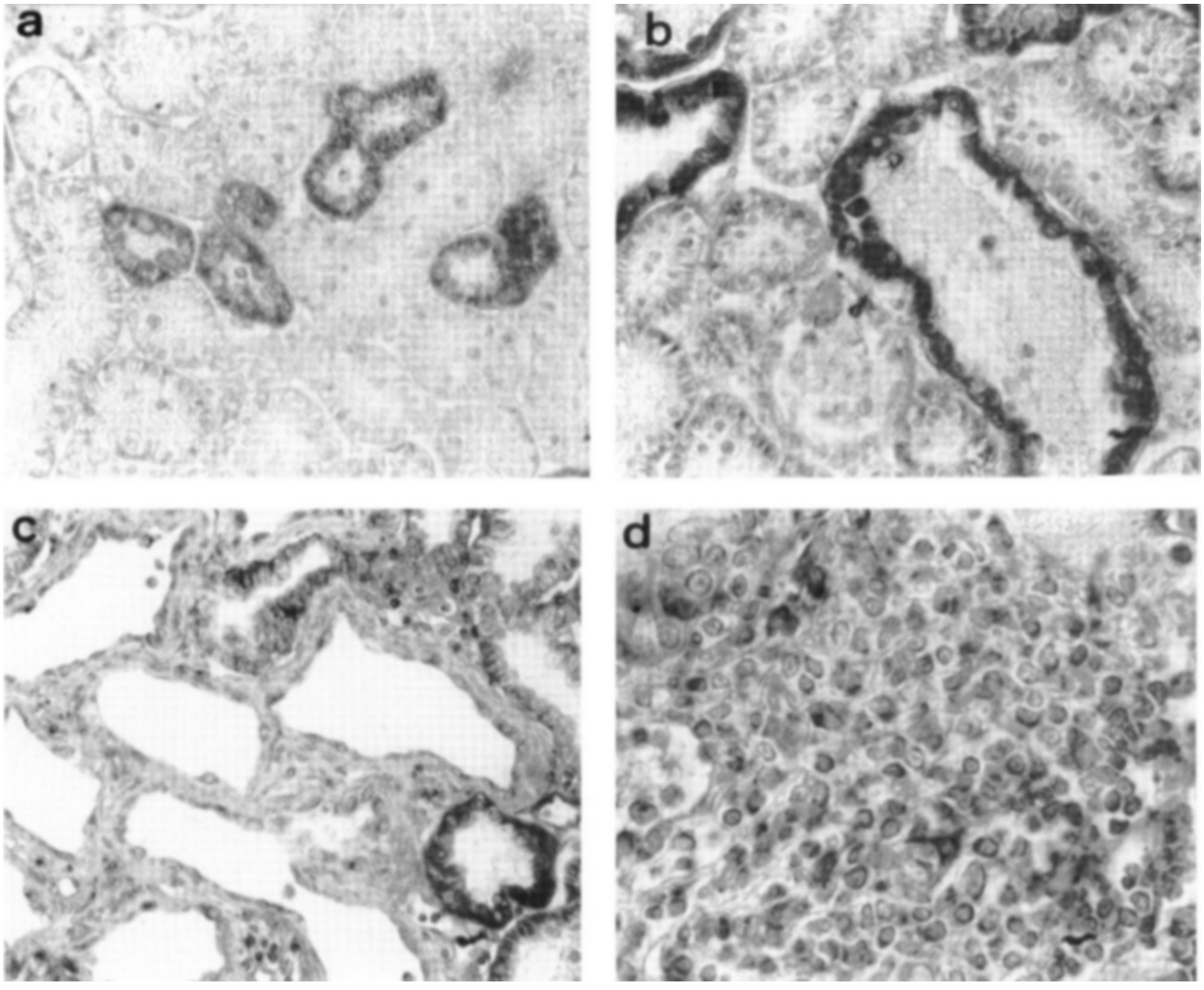


Fig. 3. *Immunostaining for PDGF B-chain.* (**a**) Sham operated controls. Variable positivity in proximal tubules and strong staining in distal tubules as well as collecting ducts (× 400). (**b**) Week 2 after 5/6 nephrectomy. Intense staining in some dilated distal tubules and/or collecting ducts. Staining is weakly positive in proximal tubules also (× 400). (**c**) Week 10 after 5/6 nephrectomy. Strong tubular staining in a dilated distal tubule and decreased staining of atrophic tubules. There also are multiple positively stained interstitial cells (× 200). (**d**) Week 7.5 after 5/6 nephrectomy. Positive immunostaining in multiple cells in an area of focal leukocytic cell infiltration (× 1000).

Growth factors: Tubulointerstitial overproduction of PDGF B-chain parallels overexpression of PDGF receptor β-subunit in areas of injury

In situ hybridization for PDGF B-chain mRNA in sham operated control rats showed focally positive signal in distal tubules and collecting ducts (Fig. 2A) as well as rare positive areas in vascular smooth muscle cells of larger vessels. This pattern was similar in control rats at three days and 10 weeks after the sham operation. Following 5/6 nephrectomy, no changes of the PDGF B-chain mRNA expression were noted at days 3, 5, and 7 after the operation. At week 2 after 5/6 nephrectomy, PDGF B-chain mRNA expression increased in distal tubules and collecting ducts and was also noted in some proximal tubules, in particular in areas of tubulointerstitial injury (Fig. 2B). In addition, interstitial cells frequently expressed the mRNA at this time point. Four weeks after 5/6 nephrectomy the tubular staining pattern for PDGF B-chain mRNA was similar to that observed at two weeks, but the interstitial expression increased further in a focal manner (Fig. 2C). Finally, at week 10 after 5/6 nephrectomy, tubular expression of PDGF B-chain mRNA had returned to normal levels, while focally increased expression was maintained in the interstitium. No significant changes of PDGF B-chain mRNA expression were noted in vessel walls after 5/6 nephrectomy. Tissue examined with a sense cRNA probe for the PDGF B-chain mRNA was negative (Fig. 2D).

Immunostaining for PDGF B-chain in sham operated controls showed variable positivity in the basolateral portions of proximal tubules and strong staining in distal tubules as well as collecting ducts at any time point examined (Fig. 3A). Positive staining was also observed in some vascular smooth muscle cells but was largely absent in interstitial cells. Following 5/6 nephrectomy, the immunohistochemical expression of PDGF B-chain mirrored that of the mRNA expression, in that no significant changes occurred prior to week 2. At week 2, immunostaining became very intense

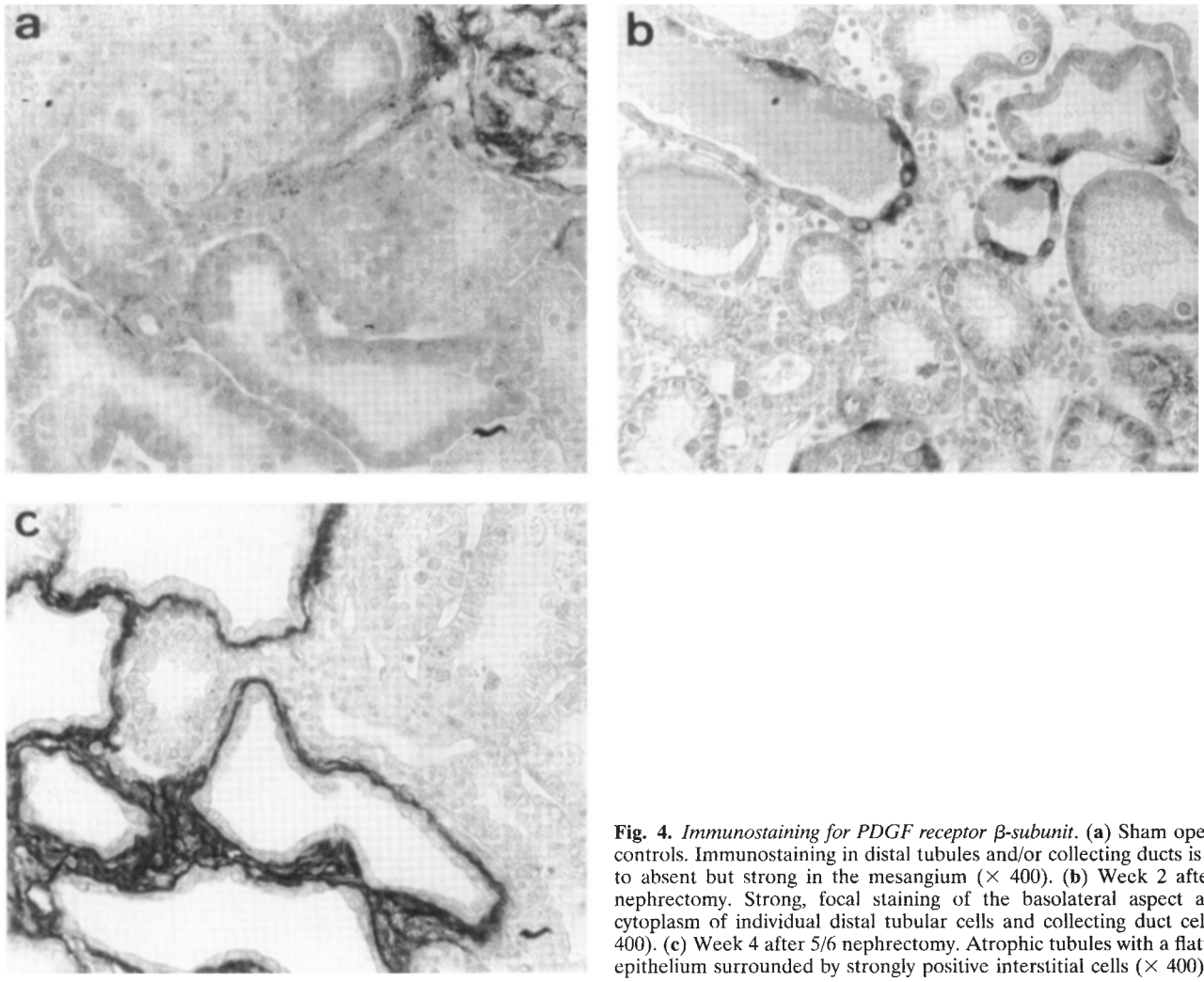


Fig. 4. *Immunostaining for PDGF receptor β-subunit.* (**a**) Sham operated controls. Immunostaining in distal tubules and/or collecting ducts is weak to absent but strong in the mesangium (× 400). (**b**) Week 2 after 5/6 nephrectomy. Strong, focal staining of the basolateral aspect and/or cytoplasm of individual distal tubular cells and collecting duct cells (× 400). (**c**) Week 4 after 5/6 nephrectomy. Atrophic tubules with a flattened epithelium surrounded by strongly positive interstitial cells (× 400).

in some distal tubules and collecting ducts, weakly positive in proximal tubules and occasionally positive in interstitial cells (Fig. 3B). Strong tubular staining in some dilated tubules and preserved distal tubules and collecting ducts then persisted until week 10, while staining of atrophic tubules decreased at the late time points (Fig. 3C). In the interstitium of 5/6 nephrectomized rats past week 2, PDGF B-chain immunostaining was mainly confined to areas of focal cell infiltrates, but could also be detected in other interstitial areas (Fig. 3 C, D). Vascular PDGF B-chain expression remained unchanged after 5/6 nephrectomy.

Immunostaining for PDGF receptor β-subunit in sham operated controls showed either absent tubulointerstitial expression or weak expression in distal tubules and/or collecting ducts as well as in vessel walls at any time point examined (Fig. 4A). First changes of this pattern were noted at week 2 after 5/6 nephrectomy, when strong, focal staining of the basolateral aspect of individual distal tubular cells and/or collecting duct cells was noted (Fig. 4B). This finding then persisted until week 10. In addition, some but not all dilated tubules, in particular atrophic tubules with a flattened epithelium, were surrounded by strongly positive interstitial cells from weeks 2 to 10 after 5/6 nephrectomy (Fig. 4C). No staining for PDGF receptor β-subunit was noted in inflammatory infiltrates and the vascular staining pattern did not change significantly after 5/6 nephrectomy.

Myofibroblasts: Progressive interstitial accumulation of cells positive for α-smooth muscle actin and desmin after 5/6 nephrectomy

In sham operated rats at any time point, interstitial expression of α-smooth muscle actin was largely confined to vessels and only rare interstitial cells lining Bowman's capsule stained positive for these cytoskeletal proteins (Figs. 5A and 6A). Immunostaining for desmin in sham operated controls showed a similar pattern, but occasional interstitial cells constitutively expressed this cytoskeletal protein (Figs. 5B and 6B). Following 5/6 nephrectomy both cytoskeletal proteins were focally overexpressed in the interstitium, starting at day 5 (α-smooth muscle actin) and week 2 (desmin) and then increased further at later time points (Figs. 5 C, D and 6 A, B). Overexpression of α-smooth muscle actin and, to a lesser degree, of desmin was particularly prominent in the periglomerular area (Fig. 5 C, D).

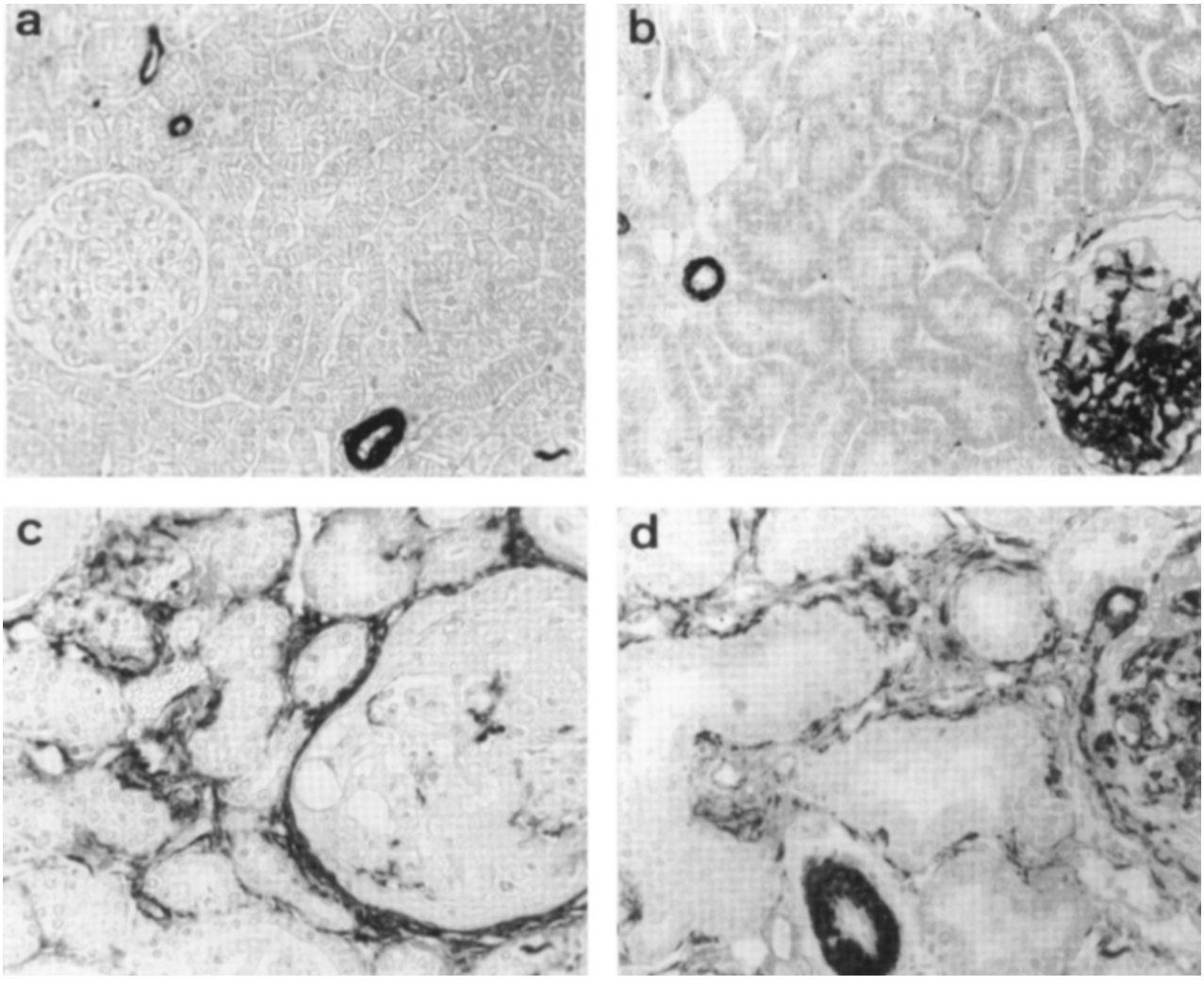


Fig. 5. *Immunostaining for α-smooth muscle actin and desmin.* (**a**) Sham operated controls. Expression of α-smooth muscle actin is confined to vessels (× 400). (**b**) Sham operated controls. Expression of desmin in vessel walls and occasional interstitial cells as well as glomeruli (×400). (**c**) Week 10 after 5/6 nephrectomy. Increased expression of α-smooth muscle actin in the tubulointerstitium. A particular concentration of positive cells has a periglomerular location (× 400). (**d**) Week 10 after 5/6 nephrectomy. Increased expression of desmin in the tubulointerstitium (× 400).

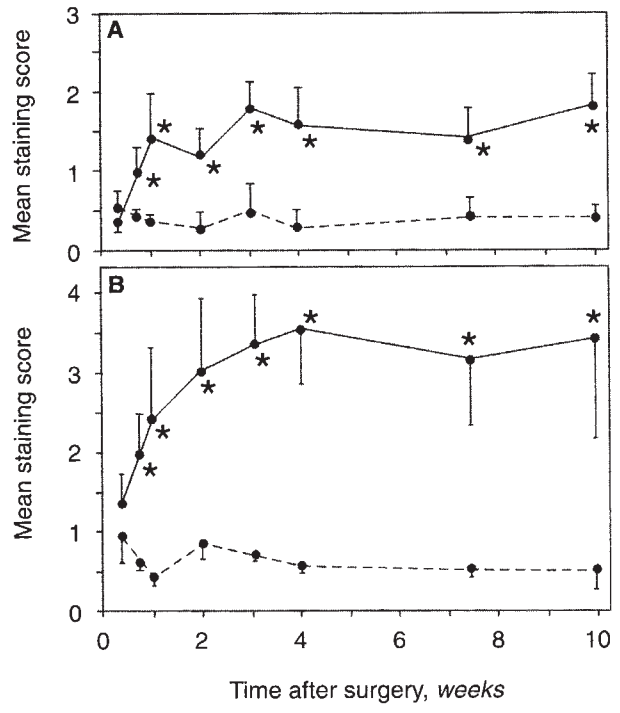
Inflammatory cells: 5/6 Nephrectomy is followed by a progressive tubulointerstitial infiltration of monocytes/macrophages

In comparison to the cortical tubulointerstitium of sham operated rats, in 5/6 nephrectomized rats an increase in monocyte/macrophage counts was noted, which started at week 2 after the operation (Fig. 7). Monocyte/macrophage counts thereafter continued to rise until the end of the observation period (Fig. 7). Monocyte/macrophage infiltration in the tubulointerstitium was focal with dense infiltrates in some areas and other relatively normal-appearing areas in all cases. In contrast to the progressive influx of monocytes/macrophages after 5/6 nephrectomy, there was only a minor and transient increase in neutrophil counts at week 2 (Fig. 7) and no increase in OX22 positive lymphocytes (data not shown) in the operated rats.

Due to their small size and their occasional occurrence in clusters, platelet counts in the cortical tubulointerstitium could not be assessed quantitatively. In sham operated rats, few platelets were detected inside small vessels or peritubular capillaries. Following 5/6 nephrectomy, no apparent change in the PL-1 immunostaining pattern was noted at any time point of the observation period (data not shown).

Matrix: Progressive interstitial accumulation of various extracellular matrix proteins after 5/6 nephrectomy

Staining for the extracellular matrix proteins type I collagen, type IV collagen, laminin, and fibronectin in sham operated controls revealed similar patterns to those described previously [20, 34]. Following 5/6 nephrectomy all four matrix proteins behaved uniformly, in that first tubulointerstitial matrix expansion was noted at week 2 after the operation. Accumulation of the matrix proteins in the cortical tubulointerstitium then increased progressively during the observation period (Figs. 8 and 9; laminin and fibronectin not shown).



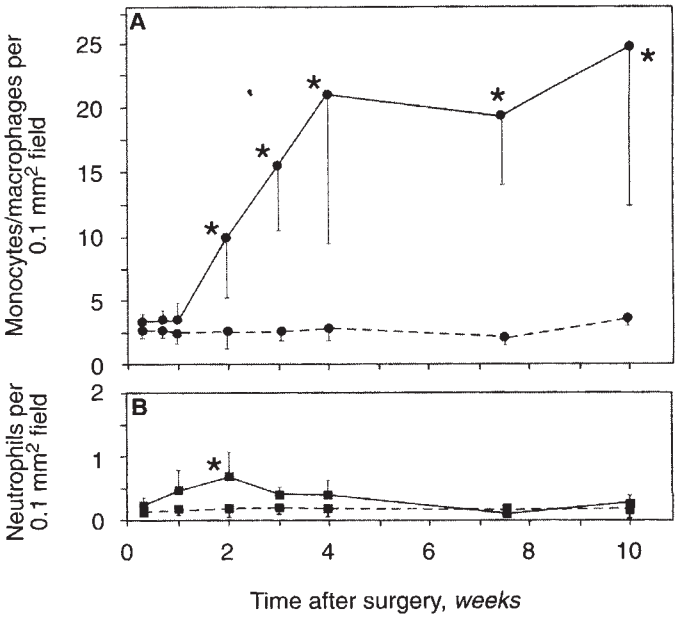


Fig. 7. *Tubulointerstitial monocytes/macrophages and neutrophils.* Quantitative assessment of tubulointerstitial monocyte/macrophage (**A**) and neutrophil (**B**) infiltration after 5/6 nephrectomy. *$P < 0.05$ versus sham operated control at the same time point.

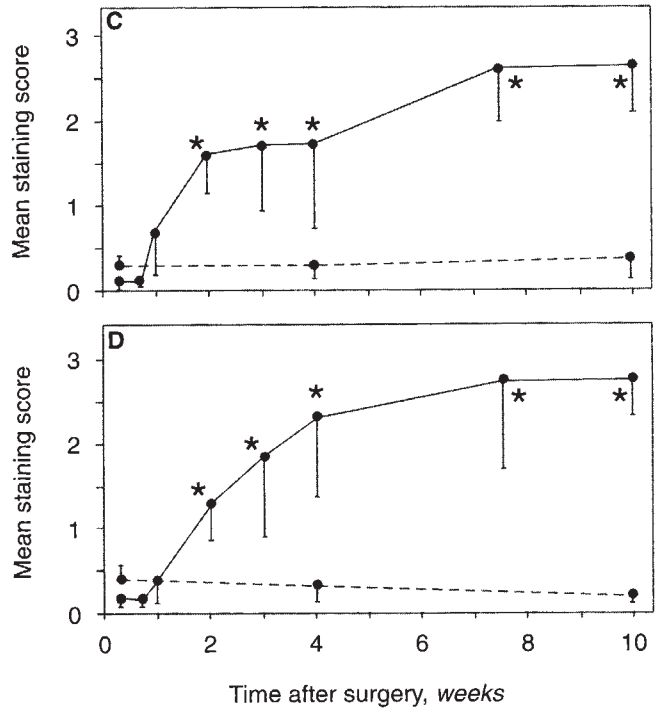


Fig. 6. *Tubulointerstitial expression of α-smooth muscle actin and desmin.* **A.** α-smooth muscle actin interstitial; **B.** α-smooth muscle actin periglomerular; **C.** Desmin interstitial; **D.** Desmin periglomerular. Semiquantitative assessment of immunostaining for α-smooth muscle actin and desmin after 5/6 nephrectomy. *$P < 0.05$ versus sham operated control at the same time point (α-smooth muscle actin) or all sham control values (desmin). Symbols are: (solid line) after 5/6 nephrectomy; (dashed line) sham operated control).

Progressive tubulointerstitial injury, proteinuria, and renal insufficiency develop past week 2 after 5/6 nephrectomy

Significant evidence of tubulointerstitial injury after 5/6 nephrectomy was first noted at week 2 and progressively increased thereafter (Table 1). Evidence of interstitial fibrosis was first detected in some biopsies obtained at week 4 after 5/6 nephrectomy, and increased again at later time points.

Indices of tubulointerstitial injury were positively correlated with immunostaining scores for interstitial and periglomerular α-smooth muscle actin (interstitial, $r = 0.65$, $P < 0.001$; periglomerular, $r = 0.61$, $P < 0.001$) and desmin (interstitial, $r = 0.82$, $P < 0.001$; periglomerular, $r = 0.77$, $P < 0.001$) as well as with tubulointerstitial monocyte/macrophage counts ($r = 0.58$, $P < 0.001$), while a negative correlation with tubulointerstitial PCNA counts was noted (tubular PCNA positive cells, $r = -0.64$, $P < 0.001$; "other PCNA positive cells," $r = -0.30$, $P < 0.07$).

A significant increase in proteinuria over that observed in sham operated rats occurred at week 2 in 5/6 nephrectomized rats. Thereafter proteinuria increased until week 7.5 (Table 1). Serum creatinine concentration was already elevated at day 3 after 5/6 nephrectomy as a consequence of the renal ablation. Creatinine values in the operated rats then remained relatively constant until week 4, after which they progressively increased (Table 1).

Discussion

The first pathological change in the cortical tubulointerstitium after 5/6 nephrectomy was a very rapid increase of cell proliferation, which was already pronounced at the earliest time point studied, that is, day 3. Interstitial cell proliferation in both tubular and non-tubular locations preceded the onset of the glomerular proliferative wave (Table 2). Similar to these findings, Miskell and

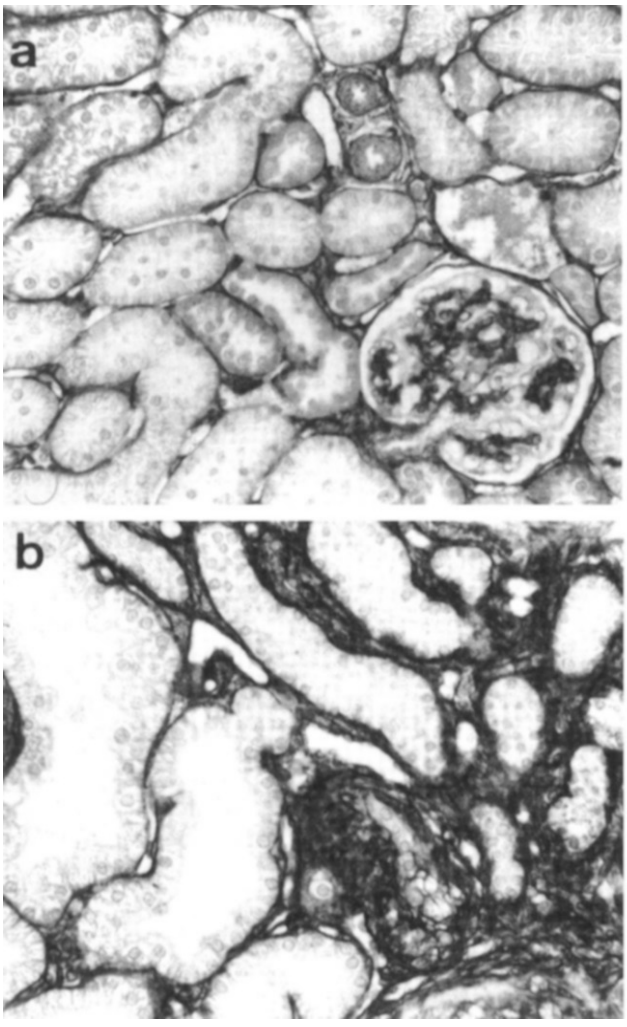


Fig. 8. *Immunostaining for type IV collagen.* (**a**) Sham operated controls. Expression of type IV collagen in tubular basement membrane, vascular walls, interstitial areas and mesangial areas (× 400). (**b**) Week 10 after 5/6 nephrectomy: Positive immunostaining for type IV collagen in the expanded interstitial matrix (× 400).

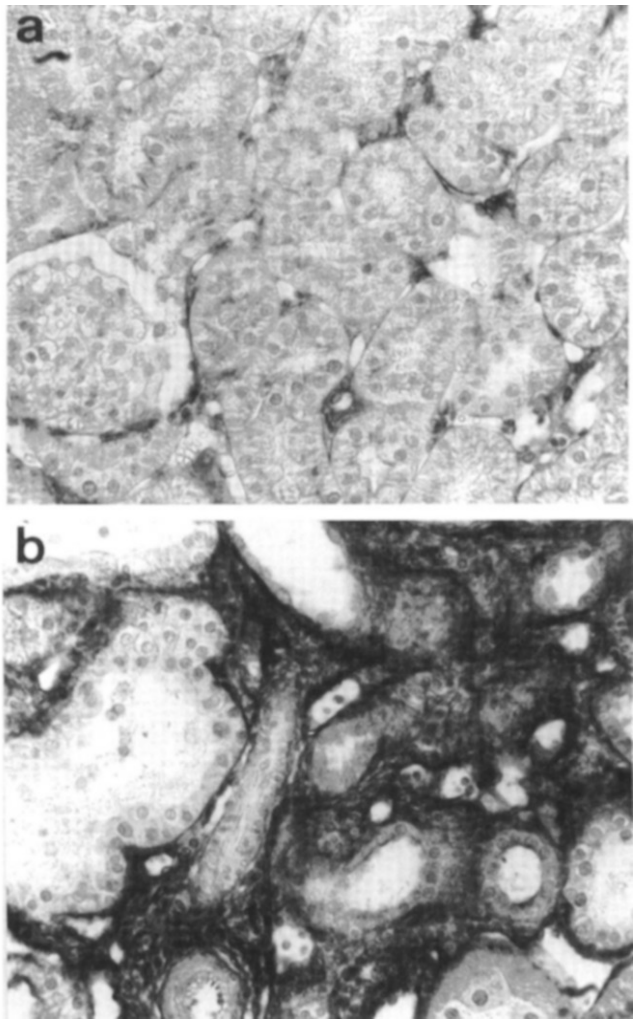


Fig. 9. *Immunostaining for type I collagen.* (**a**) Sham operated controls. Expression of type I collagen in interstitial areas (× 400). (**b**) Week 10 after 5/6 nephrectomy. Positive immunostaining for type I collagen in the expanded interstitial matrix (× 400).

Simpson [35] have shown that tubular but not glomerular cell proliferation after 5/6 nephrectomy increased within 24 hours after surgery, and that it preceded the compensatory increase in glomerular filtration rate per nephron. The present and other studies [35] have identified most of the proliferating tubular cells during the hyperplastic response as proximal tubular, although increased cell proliferation has also been noted in loops of Henle, collecting ducts, and interstitial cells. The mechanisms underlying these changes remain speculative. However, in various experimental models, tubulointerstitial proliferation was found to correlate with the caloric intake after 5/6 nephrectomy [36], and the presence of hypertension [37], angiotensin II [20, 34], and other growth factors such as epidermal growth factor, insulin-like growth factors, transforming growth factor-β (TGF-β), and ammonia [reviewed in 38]. Tubular anoxic damage and subsequent regeneration after the partial renal infarction might also account for the increased tubular proliferation rate observed in our study. However, this appears unlikely, since we obtained renal biopsies only from the center of the non-infarcted area and since we did not observe signs of tubular necrosis in any section.

In agreement with our previous data [20], constitutive PDGF B-chain expression was detected in distal tubules and collecting ducts. The present study shows that PDGF B-chain is unlikely to mediate the early tubulointerstitial hyperplasia after 5/6 nephrectomy, since neither tubulointerstitial overproduction of PDGF nor significant PDGF receptor β-subunit expression occurred before week 2 after the operation. Furthermore, *in vitro* data show that PDGF is not mitogenic for primary cultures of rabbit proximal tubular cells [39] and only weakly mitogenic for cultured rabbit renal cortical fibroblasts [40]. Finally, at least in human and primate kidney, expression of PDGF receptor β-subunit was not detected in tubular cells, albeit in some cortical interstitial cells [41–43].

During the later stages after 5/6 nephrectomy a marked up-regulation of both PDGF B-chain mRNA and protein as well as receptor β-subunit protein expression was noted in areas of

Table 1. Basic characteristics of the 5/6 nephrectomy model[a]

Week	Tubulointerstitial injury index ($N = 5$)		Urinary protein excretion *mg/24 hr* ($N = 10$)		Serum creatinine *mg%* ($N = 8$)	
	SHAM	OP	SHAM	OP	SHAM	OP
0.4	0 ± 0	0 ± 0	4 ± 2	3 ± 1	0.8 ± 0.2	1.7 ± 0.4
1	0 ± 0	0.6 ± 0.7	4 ± 1	10 ± 6	0.7 ± 0.2	1.7 ± 0.2[b]
2	0 ± 0	1.0 ± 0.6[b]	8 ± 3	38 ± 17[b]	0.8 ± 0.1	1.7 ± 0.4[b]
3	0 ± 0	2.8 ± 0.5[b]	—	—	0.7 ± 0.2	1.8 ± 0.4
4	0.2 ± 0.3	3.4 ± 0.5[b]	17 ± 6	85 ± 28[b]	0.7 ± 0.1	1.6 ± 0.3[b]
7.5	0.2 ± 0.3	3.7 ± 0.8[b]	22 ± 3	143 ± 55[b]	0.9 ± 0.2	2.2 ± 0.7[b]
10	0.3 ± 0.3	3.7 ± 0.5[b]	21 ± 3	95 ± 38[b]	1.1 ± 0.2	3.1 ± 1.3[b]

[a] Parts of this table have been published previously as part of a study on glomerular changes after 5/6 nephrectomy [7].

Data are mean ±SD. Serum-creatinine, urinary protein excretion and tubulointerstitial injury index in rats after 5/6 nephrectomy (OP) or sham-operated rats (SHAM).

[b] $P < 0.05$ vs. SHAM-group at the same time point

Table 2. Glomerular versus tubulointerstitial changes after 5/6 nephrectomy

	Week:	0.4	0.7	1	2	3	4	7.5	10
Proliferation	glomerular	→	↑	↑ ↑	↑ ↑ ↑	↑ ↑	↑ ↑	↑	↑
	tubulоint.	↑ ↑	↑ ↑	↑ ↑ ↑	↑ ↑	↑	↑	↑	→
α-Smooth muscle	glomerular	↑	↑	↑	↑	↑	↑ ↑	↑ ↑	↑ ↑
actin expression	tubulоint.	→	↑	↑	↑ ↑	↑ ↑	↑ ↑	↑ ↑	↑ ↑
Desmin expression	glomerular					not done			
	tubulоint.	→	→	→	↑	↑ ↑	↑ ↑	↑ ↑	↑ ↑
Monocytes/macrophages	glomerular	→	→	→	↑	↑	↑	↑	↑ ↑
influx	tubulоint.	→	→	→	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑	↑ ↑ ↑
PDGF B-chain	glomerular	→	→	↑	↑ ↑	↑	↑	→	→
(mRNA, protein)	tubulоint.	→	→	→	↑ ↑	↑ ↑	↑ ↑ ↑	↑ ↑	↑ ↑
PDGF receptor β-	glomerular	→	→	→	↑	↑	↑	↑	↑
subunit	tubulоint.	→	→	→	↑	↑ ↑	↑ ↑	↑ ↑	↑ ↑
Matrix protein	glomerular	→	→	→	↑	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑
accumulation	tubulоint.	→	→	→	↑	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑
Glomerulosclerosis		→	→	→	→	↑	↑	↑ ↑	↑ ↑ ↑
Tubulointerstitial injury		→	→	→	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑	↑ ↑ ↑

Semiquantitative summary of the histological glomerular changes (as published in [7 and 8]) and tubulointerstitial changes after 5/6 nephrectomy in rats.

Symbols are: (→) no change in 5/6 nephrectomized rats compared to sham operated rats; (↑, ↑ ↑, ↑ ↑ ↑) mild, moderate or marked increase in 5/6 nephrectomized rats compared to sham operated rats.

tubulointerstitial injury. In contrast, Muchaneta-Kubara and El Nahas [44] were unable to demonstrate immunohistochemical PDGF expression in rat renal interstitium of both normal and 5/6 nephrectomized rats. Since our immunohistochemical findings were independently confirmed by *in situ* hybridization, this discrepancy likely results from the usage of different antibodies to PDGF B-chain in the study of Muchaneta-Kubara and El Nahas [44]. Increased PDGF receptor β-subunit protein expression in areas of tubulointerstitial injury is in agreement with previous observations in transplanted or glomerulonephritic human kidney [41]. Finally, in angiotensin-II infused rats [20], we had also observed up-regulation of PDGF B-chain synthesis in areas of tubulointerstitial injury. However, while in this case tubulointerstitial PDGF B-chain overexpression correlated with increased proliferative activity in these areas, PDGF B-chain overexpression after 5/6 nephrectomy was dissociated from tubulointerstitial cell proliferation. While a role of PDGF in mediating the tubular cell proliferation is unlikely, it is possible that it may have mediated a low grade fibroblast proliferation. Thus, a recent study has shown that injections of large amounts of PDGF into rats can induce α-smooth muscle actin expression plus proliferation in renal interstitial cells [45]. Alternatively, the tubulointerstitial actions of PDGF B-chain may relate to its multiple non-mitogenic effects such as the chemotactic activity of PDGF B-chain for various cell types, including fibroblasts, vascular smooth muscle cells, and macrophages [reviewed in 46]. Indeed, in a vascular injury model, the main effect of a neutralizing anti-PDGF B-chain antibody was the prevention of smooth muscle cell migration into the neointima rather than the reduction of cell proliferation [47]. We therefore speculate that PDGF B-chain overproduction and increased receptor expression in areas of tubulointerstitial injury may play an important role in attracting fibrogenic cells such as fibroblasts.

Apart from cell proliferation, the expression of myofibroblast markers, such as α-smooth muscle actin and desmin, was another early change after 5/6 nephrectomy and thus closely resembled the glomerular alterations (Table 2). Increased expression of α-smooth muscle actin in the tubulointerstitium has been noted in several progressive models of renal injury [20, 34, 48]. Furthermore, we have shown that in human kidneys renal cortical fibroblasts constitutively express α-smooth muscle actin and that

this expression is up-regulated in instances of tubulointerstitial injury [49]. Others have proposed that up-regulated interstitial expression of α-smooth muscle actin in human renal biopsies may serve as a predictor of progressive renal dysfunction [50]. The mechanisms responsible for the interstitial α-smooth muscle actin overexpression remain speculative. Our study does not support an important role of macrophage-derived TGF-β in this respect, as proposed by Diamond et al [48], since α-smooth muscle actin overexpression significantly preceded the interstitial macrophage influx (Table 2). Unlike α-smooth muscle actin, the intermediate filament protein desmin was not overexpressed in either normal or diseased human renal interstitium [49]. The present study suggests that in rats transformed renal fibroblasts are able to express desmin, which is in agreement with observations in experimental hydronephrosis in rats [48]. Interstitial expression of desmin in rats may therefore be another marker of injury, which is similar to findings in the glomerulus, where desmin overexpression marks activation of mesangial cells [51] as well as podocyte injury [52].

Following the early adaptive hyperplasia and myofibroblast transformation in the tubulointerstitium after 5/6 nephrectomy, several events occurred in parallel, including an influx of monocytes/macrophages and accumulation of various extracellular matrix proteins (Table 2). All of these later changes correlated with structural and functional measures of renal damage and closely paralleled the changes observed in the glomeruli (Table 2). A close association of both glomerular and tubulointerstitial pathology with decreasing renal function has also been detected in careful morphometric studies in human mesangiocapillary glomerulonephritis [14] and diabetic nephropathy [16].

The tubulointerstitial influx of monocytes/macrophages is believed to be of central importance for the development of fibrotic changes [reviewed in 53]. This influx may have been mediated by various chemoattractants released from or expressed by injured tubular cells, including monocyte chemotactic protein-1 [54], Rantes [55], osteopontin/uropontin [56, 57], as well as overexpression of adhesion molecules such as ICAM-1 [54, 58]. The role of PDGF as a monocyte chemoattractant *in vivo* remains unknown, since despite chemotactic activity *in vitro* [46], *in vivo* transfection of glomeruli with a PDGF B-chain cDNA was not followed by a monocyte/macrophage influx [59]. From a functional aspect, macrophages appear to play an important role in mediating renal damage, since a reduction of the tubulointerstitial or glomerular macrophage influx led to markedly improved pathological findings in progressive renal injury [58, 60]. Macrophages as well as proximal tubular cells and myofibroblasts may be involved in the development of fibrosis by the release of fibrogenic peptides such as TGF-β or interleukin-1 [61–63]. These fibrogenic cytokines have been shown to stimulate the production of types I and III collagen in renal fibroblasts *in vitro* [64]. Whether their action also accounts for the observation that fibroblasts derived from fibrotic kidneys synthesize increased amounts of type III and IV collagen *in vitro* [65], remains unknown. A role of PDGF in mediating the interstitial accumulation of matrix proteins is conceivable given that: (1) interstitial fibroblasts express PDGF receptors for the B-chain [this study and 40]; (2) interstitial PDGF production and PDGF receptor expression are up-regulated during tubulointerstitial matrix accumulation; (3) PDGF is involved in the regulation of the matrix production by the myofibroblast-like glomerular mesangial cells [66]; and (4) PDGF may act as a chemoattractant for interstitial fibroblasts.

We conclude that tubulointerstitial changes after 5/6 nephrectomy show considerable similarities with those observed in the glomeruli in that an (adaptive) proliferative phase is followed by progressive tubulointerstitial injury with fibroblast transformation, inflammatory cell influx and matrix accumulation. Tubular and interstitial overexpression of PDGF B-chain and its receptor in areas of tubulointerstitial injury may play a role in mediating fibroblast migration, proliferation, and, possibly, matrix production.

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